A Sox5 gene is expressed in the myogenic lineage during trout embryonic development

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ABSTRACT  Sox proteins form a family of transcription factors characterized by the presence of a DNA-binding domain called the high-mobility-group domain (HMG). The presence of a large number of potential Sox5 binding sites in the trout promoter of Pax7, a gene which has emerged as an important regulator of neural and somite development, prompted us to clone trout Sox5 and to examine its expression pattern in the developing trout embryo. Using whole mount in situ hybridisation, we show here that the Sox5 transcript is first expressed before segmentation in the whole presomitic mesoderm. In newly formed somites, Sox5 labelling was observed in myogenic progenitor cells of the posterior and anterior walls. As the somite matured rostrocaudally, Sox5 expression disappeared from the differentiating embryonic myotome, deep in the somite, to become restricted to the undifferentiated myogenic precursors forming the dermomyotome-like epithelium at the surface of the embryonic myotome. Sox5 was also expressed in the developing nervous system and in pectoral fin buds. On the whole, this work suggests a hitherto unappreciated role for Sox5 in regulating myogenic cells destined to muscle formation and growth.

KEY WORDS: Sox5, somite, myotome, external cell layer, dermomyotome, teleost

Introduction

Sox proteins are transcription factors which contain a DNA-binding domain called the high-mobility-group domain (HMG). They bind to the minor groove of DNA and recognize the consensus motif (A/T)(A/T)CAA(A/T) (Mertin et al. 1999). Several Sox proteins have been shown to play major role in vertebrate development including early embryogenesis, gastrulation and neural induction, and to contribute to differentiation in many lineages (Guth and Wegner, 2008). Sox proteins are categorized into several subgroups on the basis of sequence similarity within the HMG box and other domains (Schepers et al. 2002).

Myogenic differentiation is controlled by a complex transcriptional regulatory network involving the four myogenic regulatory factors (MRFs) MyoD, myf5, myogenin and MRF4, which direct the transcription of muscle structural genes (Buckingham, 1992). MRFs act downstream of, or in parallel with, the paired domain and homeobox-containing transcription factors Pax3 and Pax7 which are precociously expressed in the dermomyotome, a somite derivative from which arise the myogenic precursors necessary for embryonic myotome development and muscle growth (Buckingham and Relaix, 2007). An external cell layer of undifferentiated Pax-7 positive myogenic cells surrounds the primary myotome in the fish embryo. This external cell layer, that exhibits many features of the amniote dermomyotome (Devoto et al. 2006), derives from the anterior somitic domain by a cell arrangement and provides myogenic cell precursors necessary for mediolateral expansion of the embryonic myotome (Hollway et al. 2007; Stellabotte et al. 2007; Steinbacher et al. 2008).

As with MRF and Pax7 genes, several Sox genes have been shown to regulate muscle differentiation. Thus Sox8 and Sox15 are expressed in muscle satellite cells and are down-regulated during myogenic differentiation (Schmidt et al. 2003; Beranger et al. 2000). Mice lacking Sox15 are viable but appear to have impaired skeletal muscle regeneration (Lee et al. 2004). On the other hand, Sox6 has been shown to play a critical role in the fiber type differentiation of skeletal muscle both in both the mouse and fish. Indeed, all fetal muscle fibers in Sox6 null mouse embryos

Abbreviations used in this paper: HMG, high mobility group (domain); MRF, myogenic regulatory factor.

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maintain slow fiber characteristics (Hagiwara et al. 2007), while forcing the expression of Sox6 in zebrafish embryo adaxial cells inhibits the expression of Prox1, a gene involved slow fiber maturation (Hofsten et al. 2008).

Sox5 belongs to the SoxD subgroup together with Sox6 and Sox13 and is primarily expressed in cartilage, its expression being associated with the chondrocyte phenotype (Lefebvre et al. 1998). Sox5 transcript has also been found in many adult tissues, including the brain, kidney, lung and skeletal muscle (Ikeda et al. 2002). The identification of several potential Sox5-binding sites in the promoter of Pax7, which is a major developmental gene, led us to analyze the expression of Sox5 in trout embryos, particularly in relation with somite development.

Results and Discussion

The trout Pax7 proximal promoter contains several Sox5 binding sites

In an attempt to identify genes precociously expressed in myogenic progenitor cells of the trout embryo we searched for...
Sox5 expression in the myogenic lineage of trout embryo

Molecular cloning of trout Sox5 cDNA

All the PCR products generated in course of this study were overlapping and resulted in a 2289 nt cDNA (GeneBank accession no. FJ713023) with an open reading frame encoding a 762 amino acid protein (Fig. 1). The BlastP best match of this protein was zebrafish Sox5. The identity of putative trout Sox5 protein was further confirmed using the Figenix platform which unambiguously clustered it with Sox5 proteins within the SoxD subgroup (fig. 2).

Expression pattern of Sox5 in developing trout embryo

Whole mount in situ hybridisation using an antisense Sox5 digoxigenin-labeled probe showed that Sox5 transcript was first present in two presomitic bands prior to the onset of somitogenesis (stage 10A of Ballard, 1973) (Fig. 3A). At the beginning of the somitogenesis, 6-10 somites formed simultaneously in the rostral paraxial mesoderm (stage 10B). At this stage Sox5 transcript was observed within the elongating presomitic mesoderm and in somites. As somitogenesis proceeded along an anteroposterior axis (stages 10B to 19), labelling extended progressively to more caudal somites (Fig. 3B,C). Transverse sections showed that Sox5 transcript was present throughout the rostral presomitic mesoderm including adaxial and lateral cells (Fig. 5A). In the newly formed somites, Sox5 labelling appeared to be intense in a central stripe (Fig. 3B). Frontal sections indicated that this aspect resulted from the marked accumulation of Sox5 transcript in the cytoplasm of myogenic cell precursors of the posterior and anterior walls and its weak accumulation in the large nuclei mostly located at the apical pole of these cells (Fig 4A,B). Shortly after their incorporation into somite, the adaxial cells which had already started to express myogenin (Fig. 4C) ceased to express Sox5 (Fig. 4B). It is now established that adaxial cells, which are the first to differentiate, migrate radially through the somite to form a superficial layer of embryonic slow muscle fibres covering the fast embryonic muscle fibres that originate from the posterior somitic compartment (Devoto et al. 1996; Hollway et al. 2007; Stellabotte et al. 2007). As somite maturation proceeded, Sox5 expression gradually disappeared from the differentiating embryonic myotome, deep in the somite (Fig. 5B), to become restricted to the surrounding external cell layer (Fig. 5 C,D). This external epithelium, produced by the displacement of somitic anterior cells towards the outermost part of the somite (Hollway et al. 2007; Stellabotte et al. 2007), exhibits many features of the amniote...
dermomyotome, including the expression of equivalent gene orthologs (Devoto et al. 2006; Dumont et al. 2008) and the ability to provide myogenic cells for myotome expansion (Hollway et al. 2007; Stellabotte et al. 2007; Steinbacher et al. 2008).

That Sox5 expression faded in differentiating cells of the primary myotome while being maintained in somitic external cells indicated that Sox5 expression is related to an undifferentiated state of the embryonic myogenic cells. In line with this observation, it is interesting to note that Sox8 and Sox15 are also expressed in mouse myogenic cells prior to their differentiation into myotubes, and have been found to inhibit myogenesis (Beranger et al. 2000; Schmidt et al. 2003). At the end of somitogenesis, Sox5 labelling appeared in pectoral fin buds which at this stage are evident as MRF-negative oval structures budding from the ventral portion of rostral somites (Macqueen and Johnston, 2008). An expression of Sox5 appeared in brain structures and in few cell subpopulations of the neural tube around the 20 somite stage (Fig. 5A). At the end of somitogenesis, Sox5 transcript was evidenced in the cerebellum, the optic tectum, the telencephalon, the lens, the retina, and branchial arches (Fig. 3C). In mouse embryo Sox5 has been shown to be expressed in notochord cells and in sclerotome cells surrounding the notochord and neural tube (Smits and Lefebvre, 2003). The Sox5 gene identified in this study was not expressed in notochord and sclerotome cells of the trout embryo (Fig. 5 B,C,D). Given the ancient whole-genome duplication that occurred in the teleost fish lineage, after split of lobe and ray finned lineages (Jaillon et al. 2004), we cannot exclude that an additional Sox 5 gene copy with an expression in the forming vertebral column exists in the trout genome. Alternatively, the acquisition of a chondrogenic function by Sox5 may be specific to mammal evolution.

In conclusion, and on the basis of the presence of multiple potential Sox5 binding sites on the promoter of the Pax7 developmental gene, we examined the expression pattern of Sox5 in developing trout embryos. The expression data showed that Sox5 was transcribed in somite myogenic precursors giving rise to the embryonic myotome and in those forming the dermatomyotome-like epithelium at the surface of the embryonic myotome. This work thus suggests a hitherto unappreciated role for Sox5 in regulating myogenic progenitor cells destined to muscle formation and growth.

Materials and Methods

Fish maintenance

All experiments were carried out on the rainbow trout *Oncorhynchus mykiss* (Walbaum). Eggs were collected at the experimental facilities of the INRA Drennec fish farm (Finistère, France). After artificial insemination, the eggs were incubated at 10°C in recirculating dechlorinated water. Chemical water parameters were regularly monitored. Oxygen levels were always above 98% saturation.

Fig. 5. Sox5 somitic expression gradually becomes restricted to external cells. (A) Stage 25 somite embryo, transverse section through the anterior presomitic mesoderm. (B) Stage 55-somite embryo, transverse section through posterior trunk. (C) Eyed-stage trout embryo, transverse section through posterior trunk. (D) Higher magnification of (C); Sox5 transcript is restrictedly present in the external cell layer situated at the outermost domain of the somite (arrow) just beneath the epidermis (arrowhead). Scale bars: 50 μm in (A,B,C) and 30 μm in (D).
BAC library screening and sequencing of the 5'-flanking regulatory region of Pax7

To isolate the trout Pax7 promoter, a 5.3X genome coverage rainbow trout bacterial artificial chromosome (BAC) library (Palti et al. 2004) was screened by PCR using primers designed from the salmon Pax7 cDNA sequence (Gottensparre et al. 2006). DNA from positive BACs was isolated using the Nucleobond BAC Maxi kit (BD Bioscience). The primer walking method was then used to obtain a genomic sequence directly from the selected BACs. The samples were sequenced using an automatic sequencing system (ABI Prism 310, PE Biosystems). Sequence analysis of the 5 regulatory region was performed with the on-line program TRES (transcription regulatory element search: http://bioportal.bioc.nus.edu.sg/tres/) using transfect weight matrices (Wingender et al 2000) with a stringent matrix cut-off score >99.

Isolation of trout Sox5 cDNA

Two rainbow trout ESTs (CX252793 and CX 252794) were selected after a tblastn search using the zebrafish (CAXK04950) Sox5 sequence. The sequencing of the corresponding clone and blastx analysis led to the identification of a cDNA fragment (position 1135-2082 in the full length sequence (GeneBank accession no. FJ713023)) encoding a partial protein sequence highly related to Sox5. The missing 5' part of the cDNA was amplified from a rainbow trout embryonic cDNA libraries (Uni-ZAP custom cDNA Library; Stratagene, La Jolla, CA) using the reverse primer ATGCAGCAGGCTGACCTGAC (position: 1336-1312) and the forward primer ATGACAGCAGGTCTATGGAGCTGAC (position: 1312-2005) of the group D, to which Sox5 belongs (Koopman et al. 2004), were isolated using the Nucleobond BAC Maxi kit (BD Bioscience). The primer

Whole-mount in situ hybridisation

Embryos were dechorionated with fine forceps and fixed overnight at 4°C in paraformaldehyde in phosphate buffered saline (PBS). The specimens were then dehydrated and stored in methanol at -20°C. After rehydration in graded methanol-PBS baths, the embryos were processed according to established automated procedures. Hybridizations were performed with Sox5 and myogenin digoxigenin-11UTP- labelled antisense riboprobes. Myogenin riboprobe was complementary to the 3' untranslated region and the 3' two thirds of the coding sequence of the trout myogenin transcript (Rescan et al. 1995). Sox5 antisense RNA probes was synthesised from a PCR-amplified template that included the HMG domain (position 1136-2082 in the full length sequence). This Sox5 riboprobe did not cross-hybridise with its closest paralog Sox6 as shown by the distinct expression pattern of Sox6 (data not shown).

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